SPECIFICATION

NOVEL CARBONYL REDUCTASE, GENE THEREOF AND METHOD OF USING
THE SAME

TECHNICAL FIELD

The present invention relates to a novel polypeptide,
a gene coding for the polypeptide, an expression vector for
the expression of the polypeptide, a transformant obtained
by transformation of a host using the expression vector,
and a production method of a compound useful as a material
for the synthesis of medicinal and other compounds using
the above transformant.

In more detail, the invention relates to a
polypeptide isolated from a microorganism having enzyme
activity in asymmetrically reducing N-benzyl-3-
pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol and
having such enzyme activity, a DNA coding for the
polypeptide, an expression vector containing the DNA, and a
transformant obtained by transformation using the
expression vector. The present invention also relates to a
production method of (S)-N-benzyl-3-pyrrolidinol.

(S)-N-Benzyl-3-pyrrolidinol is a compound useful as
an intermediate for the synthesis of medicinal compounds
such as β-lactam antibiotics and dihydropyridine compounds.

BACKGROUND ART

Known as the production method of optically active
(S)-N-benzyl-3-pyrrolidinol are the method which comprises
synthesizing from an optically active compound and the
method which comprises carrying out asymmetric synthesis or
optical resolution starting with a prochiral compound. As
such a method, JP-A-06-141876 discloses a production method

of optically active N-benzyl-3-pyrrolidinol which comprises
stereoselectively reducing N-benzyl-3-pyrrolidinone in the
presence of an enzyme having activity in stereoselectively
reducing this N-benzyl-3-pyrrolidinone. Further, JP-A-10-
5 150997 discloses a production method of optically active N-
benzyl-3-pyrrolidinol which comprises treating N-benzyl-3-
pyrrolidinone with a cell or a culture of a microorganism
or a treated product thereof. However, these methods are
low in attainable substrate concentration and in conversion
10 from the substrate to the product, hence cannot be put to
practical use.

SUMMARY OF THE INVENTION

The present inventors found a microorganism-derived
15 polypeptide which asymmetrically reduces N-benzyl-3-
pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol and
found that (S)-N-benzyl-3-pyrrolidinol can be produced
efficiently, and have now completed the present invention.

It is an object of the present invention to provide a
20 polypeptide capable of asymmetrically reducing N-benzyl-3-
pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol.
Another object of the invention is to produce that
polypeptide efficiently utilizing the gene recombination
technology. A further object of the invention is to
25 provide a transformant capable of simultaneously producing,
at high levels, that polypeptide and a polypeptide having
glucose dehydrogenase activity and, further, provide a
practical production method of (S)-N-benzyl-3-pyrrolidinol
using that transformant.

30 Thus, the present invention comprises a polypeptide
having the following physicochemical properties (1) to (5):
(1)   Action: It asymmetrically reduces N-benzyl-3-
pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol with

NADPH as a coenzyme;

(2)　　Optimum action pH:　4.5 to 5.5;

(3)　　Optimum action temperature:　40°C to 45°C;

(4)　　Molecular weight:　About 29,000 as determined by gel filtration analysis, about 35,000 as determined by SDS-polyacrylamide gel electrophoresis analysis;

(5)　　Inhibitor:　It is inhibited by the divalent copper ion.

Further, the present invention is a polypeptide described in the following (a) or (b):

(a)　　A polypeptide having the amino acid sequence shown under SEQ ID NO:1 in the sequence listing;

(b)　　A polypeptide having an amino acid sequence obtainable from the amino acid sequence shown under SEQ ID NO:1 in the sequence listing by substitution, insertion, deletion and/or addition of one or more amino acids and

having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol.

Furthermore, the present invention comprises DNAs coding for these polypeptides. Or, it also comprises a DNA coding for a polypeptide having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol, and hybridizing with a DNA having a nucleotide sequence shown under SEQ ID NO:2 in the sequence listing under stringent conditions, or a DNA coding for a polypeptide having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol, and having at least 60% sequence identity with a nucleotide sequence shown under SEQ ID NO:2 in the sequence listing.

Furthermore, it comprises an expression vector containing any of these DNAs and a transformant containing

such expression vector.

The present invention also comprises a production method of (S)-N-benzyl-3-pyrrolidinol comprising

a step of reacting such transformant and/or a treated product thereof with N-benzyl-3-pyrrolidinone, and

a step of harvesting the thus-produced (S)-N-benzyl-3-pyrrolidinol.

DETAILED DISCLOSURE OF THE INVENTION

In the following, the present invention is described in detail.

First, the polypeptide of the invention is described.

The polypeptide of the invention has enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone represented by the formula (I) shown below to produce (S)-N-benzyl-3-pyrrolidinol represented by the formula (II) shown below.
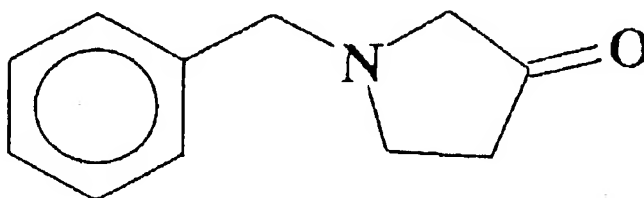
(I)

N-Benzyl-3-pyrrolidinone

5
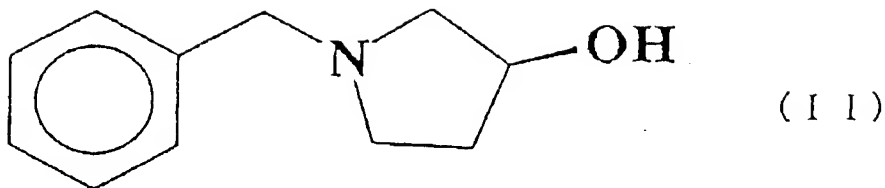
(S)-N-Benzyl-3-pyrrolidinol   (I I)

As such polypeptide, there may be mentioned an enzyme
having the following physicochemical properties (1) to (5).
(1)   Action: It asymmetrically reduces N-benzyl-3-
5 pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol with
NADPH as a coenzyme;
(2)   Optimum action pH: 4.5 to 5.5;
(3)   Optimum action temperature: 40°C to 45°C;
(4)   Molecular weight: About 29,000 as determined by gel
10 filtration analysis, about 35,000 as determined by SDS-
polyacrylamide gel electrophoresis analysis;
(5)   Inhibitor: It is inhibited by the divalent copper
ion.

        In the present invention, the enzyme activity of the
15 polypeptide is determined by adding the substrate N-benzyl-
3-pyrrolidinone (1 mM), the coenzyme NADPH (0.167 mM) and
the enzyme to 100 mM phosphate buffer (pH 6.5) and
measuring the decrease in absorbance at the wavelength 340
nm at 30°C.

20   The optimum action pH and optimum action temperature
of the peptide are determined, for example by varying the
reaction pH or reaction temperature in the above reducing
activity measurement system and measuring the reducing
activity.

25   The gel filtration analysis-based molecular weight of

the peptide is determined by calculation from the elution
time relative to those of reference proteins in gel
filtration. The SDS-polyacrylamide gel electrophoresis-
based molecular weight is determined by calculation from

5   the mobility relative to those of reference proteins in
SDS-polyacrylamide gel electrophoresis.

    The inhibitors are determined, for example by adding
various compounds to the above reducing activity
measurement system and measuring the reducing activity of

10   each compound.

    The polypeptide of the invention can be obtained from
a microorganism having an activity in asymmetrically
reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-
3-pyrrolidinol. Thus, the microorganism to be used as the

15   source of the polypeptide is not particularly restricted
but includes, for example, microorganisms belonging to the
genus Micrococcus, among which the strain Micrococcus
luteus IFO 13867 is particularly preferred. The
microorganism producing the polypeptide of the invention

20   may be a wild strain or a mutant. Or, a microorganism
obtainable by cell fusion or by such a genetic method as
gene manipulation may also be used. A gene manipulated
microorganism capable of producing the polypeptide of the
invention can be obtained by a method comprising a step of

25   isolating and/or purifying such enzyme and determining a
part or the whole of the amino acid sequence thereof, a
step of determining a nucleotide sequence coding for the
enzyme based on that amino acid sequence, a step of
obtaining a nucleotide sequence coding for the enzyme based

30   on that amino acid sequence, and a step of obtaining a
recombinant microorganism by introducing that nucleotide
sequence into another microorganism.

    As for the culture medium for the microorganism

producing the polypeptide of the invention, ordinary liquid culture medium containing carbon sources, nitrogen sources, inorganic salts, organic nutrients and so on can be used provided that the microorganism can grow thereon.

5   The term "culture of the microorganism" as used in this specification means cells of the microorganism or a culture fluid containing such cells. The "treated product thereof" means an extract or purified product obtained from the cells of the microorganism or a culture fluid

10 containing such cells by extraction, purification or some other treatment.

The polypeptide of the invention can be purified from the microorganism producing that polypeptide in the conventional manner. For example, cells of the

15 microorganism are cultured on an appropriate medium, and cells are harvested from the culture fluid by centrifugation. The cells obtained are disrupted using a sonicator, for instance, and the cell residue is removed by centrifugation to give a cell-free extract. The

20 polypeptide can be purified from this cell-free extract by applying, singly or in combination, such techniques as salting out (e.g. ammonium sulfate precipitation, sodium phosphate precipitation), solvent precipitation (protein fractionation precipitation using acetone, ethanol or the

25 like), dialysis, gel filtration, ion exchange, column chromatography such as reversed phase and ultrafiltration.

The polypeptide of the invention may be a natural enzyme obtained from a microorganism as mentioned above or may be a recombinant enzyme. As a natural enzyme, there

30 may be mentioned a polypeptide having the amino acid sequence shown under SEQ ID NO:1 in the sequence listing.

The polypeptide of the invention may also be a polypeptide having an amino acid sequence obtainable from

the amino acid sequence shown under SEQ ID NO:1 in the sequence listing by substitution, insertion, deletion and/or addition of one or more amino acids and having enzyme activity in asymmetrically reducing N-benzyl-3-

5 pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol.

Such polypeptide can be prepared from the polypeptide having the amino acid sequence shown under SEQ ID NO:1 in the sequence listing by such a known method as described in Current Protocols in Molecular Biology (John Wiley and Sons,

10 Inc., 1989).

The phrase "having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol" is used herein to indicate that when the polypeptide in question is subjected to reaction with N-

15 benzyl-3-pyrrolidinone under the above-mentioned reducing activity measurement conditions, (S)-N-benzyl-3-pyrrolidinol is produced in a yield not less than 10%, preferably not less than 40%, more preferably not less than 60%, as compared with the case where the polypeptide having

20 the amino acid sequence shown under SEQ ID NO:1 in the sequence listing is used.

The DNA of the invention is described in the following.

The DNA of the invention may be any DNA coding for

25 such a polypeptide as mentioned above. It may be a DNA having the nucleotide sequence shown under SEQ ID NO:2 in the sequence listing, or a DNA coding for a polypeptide having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol, and

30 hybridizing with the DNA having the nucleotide sequence shown under SEQ ID NO:2 in the sequence listing under stringent conditions.

The term "DNA hybridizing with the DNA having the

nucleotide sequence shown under SEQ ID NO:2 in the sequence listing under stringent conditions" means a DNA obtainable by the technique of colony hybridization, plaque hybridization or southern hybridization, using the DNA

5 having the nucleotide sequence shown under SEQ ID NO:2 in the sequence listing as a probe. More specifically, there may be mentioned a DNA identified by carrying out hybridization using a filter with the colony or plaque-derived DNA immobilized thereon, at 65°C in the presence of

10 0.7 to 1.0 M NaCl, and then washing the filter with a 0.1- to 2-fold concentrated SSC solution (1-fold concentrated SSC solution comprising 150 mM sodium chloride and 15 mM sodium citrate) at 65°C.

The hybridization can be carried out according to the

15 method described in Molecular Cloning, A laboratory manual, second edition (Cold Spring Harbor Laboratory Press, 1989) or elsewhere.

The DNA of the invention may be a DNA coding for a polypeptide having enzyme activity in asymmetrically

20 reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol and having at least 60% sequence identity, preferably at least 80% sequence identity, more preferably at least 90% sequence identity, still more preferably at least 95% sequence identity, most preferably at least 99%

25 sequence identity, with the nucleotide sequence shown under SEQ ID NO:2 in the sequence listing.

The term "sequence identity" means that the two nucleotide sequences under comparison are identical with each other, and the percentage (%) of sequence identity

30 between two nucleotide sequences under comparison is calculated by optimally arranging the two nucleotide sequences under comparison, counting those positions at which the same nucleotide (e.g. A, T, C, G, U or I) appears

in both the sequences, dividing the thus-found number of conforming positions by the total number of bases under comparison and multiplying the quotient by 100. The sequence identity can be calculated using the following tools for sequence analysis: Unix Base GCG Wisconsin Package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive Madison, Wisconsin, USA 53711; Rice, P. (1996) Program Manual for EGCG Package, Peter Rice, The Sanger Centre, Hinxton Hall, Cambridge, CB10 1RQ, England) and the ExPASy World Wide Web Molecular Biology Server (Geneva University Hospital and University of Geneva, Geneva, Switzerland).

The DNA of the invention can be obtained from a microorganism having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol. As the microorganism, there may be mentioned, for example, microorganisms belonging to the genus Micrococcus and, as a particularly preferred strain, there may be mentioned the strain Micrococcus luteus IFO 13867.

In the following, an embodiment of the method of obtaining the DNA of the invention from a microorganism having enzyme activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol is described.

First, partial amino acid sequence of the purified polypeptide and of peptide fragments obtainable by digestion of that polypeptide with an appropriate endopeptidase are determined by the Edman technique. DNA primers are synthesized based on the thus-obtained amino acid sequence information. Then, the chromosomal DNA of the microorganism is prepared from that microorganism,

which is the source of the above DNA, by a conventional
method of DNA isolation, for example by the method of
Murray et al. (Nucl., Acids Res. 8:4321-4325 (1980)).
Using the above DNA primers, PCR is carried out with the
5 chromosomal DNA as the template to amplify part of the
polypeptide gene. Further, DNA probes are prepared by
labeling part of the thus-amplified polypeptide gene by
conventional methods, for example by the random primer
labeling method (Anal. Biochem., 132, 6 (1983)). The
10 chromosomal DNA of the microorganism is cleaved with an
appropriate restriction enzyme, the restriction enzyme
cleaved fragments are inserted into a vector and the
resulting vectors are introduced into appropriate host
cells to thereby construct a DNA library of the microbial
15 chromosome. Screening of this DNA library is carried out
by the colony hybridization, plaque hybridization or like
method using the above DNA probes, whereby a DNA containing
the polypeptide gene can be obtained. The nucleotide
sequence of the thus-obtained DNA fragment containing the
20 polypeptide gene can be determined by the dideoxy
sequencing method or dideoxy chain termination method, or
the like. For example, this can be carried out using the
ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction
Kit (product of Perkin-Elmer) and the ABI 373A DNA
25 Sequencer (product of Perkin-Elmer).

The expression vector and transformant of the present
invention are now described.

The enzyme gene can be expressed in the transformant
which is obtainable by inserting the DNA of the present
30 invention to a vector and introducing the vector into a
host. The vector to be used for this purpose may be any of
those capable of expressing the enzyme gene in appropriate
hosts. As such vector, there may be mentioned, for example,

a plasmid vector, phage vector, cosmid vector, etc. It may be a shuttle vector capable of gene exchange between different host strains. Generally, such vector comprises such a regulatory factor as the lac UV5 promoter, trp promoter, trc promoter, tac promoter, lpp promoter, tuf B promoter, rec A promoter or pL promoter, and is suitably used as an expression vector containing an expression unit operatively connected to the DNA of the invention.

The term "regulatory factor" as used herein means a functional promoter and a nucleotide sequence having arbitrary related transcription elements (e.g. enhancer, CCAAT box, TATA box, SPI site, etc.).

The term "operatively connected" as used herein means that the DNA and various regulatory elements, such as promoter and enhancer, which regulate the expression thereof are joined together each in a state operative in a host so that the gene can be expressed. It is well known to the artisan that the type and species of the regulatory factor may vary according to the host.

As the host into which an expression vector containing the DNA of the invention is to be introduced, there may be mentioned bacteria, yeasts, filamentous fungi, plant cells and animal cells, for instance. Escherichia coli is preferred, however. The DNA of the invention can be introduced into a host in the conventional manner. When Escherichia coli is used as the host, the DNA of the invention can be introduced into the same by the calcium chloride method, for instance.

For producing (S)-N-benzyl-3-pyrrolidinol by asymmetrically reducing N-benzyl-3-pyrrolidinone using the DNA of the invention, a coenzyme such as NAPDH or NADH is required. However, by carrying out the reaction using an enzyme capable of converting the coenzyme oxidized to its

reduced form (hereinafter referred to as coenzyme regenerating ability) together with a substrate thereof, namely combining a coenzyme regeneration system with the polypeptide of the invention, it is possible to markedly

5 reduce the consumption of the coenzyme, which is expensive. Usable as the enzyme having coenzyme regenerating ability are, for example, hydrogenase, formate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, glucose-6-phosphate dehydrogenase and glucose dehydrogenase. Glucose

10 dehydrogenase is suitably used.

When a transformant containing both the DNA of the invention and a DNA coding for a polypeptide having glucose dehydrogenase activity is used, the above reaction can be carried out efficiently without separately preparing an

15 enzyme having coenzyme regenerating ability and adding the same to the reaction system, although such reaction may also be carried out by adding a coenzyme regeneration system to the asymmetric reduction reaction system. Such transformant can be obtained by inserting the DNA of the

20 invention and a DNA coding for a polypeptide having glucose dehydrogenase activity into the same vector and introducing this into a host, or by inserting these two DNAs respectively into two different vectors belonging to incompatible groups and introducing these into the same

25 host. Thus, a transformant containing an expression vector comprising the DNA of the invention and the DNA coding for a polypeptide having glucose dehydrogenase activity, or a transformant containing both a first expression vector containing the DNA of the invention and an expression

30 vector containing the DNA coding for a polypeptide having glucose dehydrogenase activity can be used. As for the polypeptide having glucose dehydrogenase activity, Bacillus megaterium- derived one is preferred.

The glucose dehydrogenase activity in the transformant is determined by adding the substrate glucose (0.1 M), the coenzyme NADP (2 mM) and the enzyme to 1 M Tris hydrochloride buffer (pH 8.0) and measuring the
5　increase in absorbance at the wavelength 340 nm at 25°C.

Now, a production of (S)-N-benzyl-3-pyrrolidinol using the transformant of the invention is described.

Such production method comprises a step of reacting the above transformant and/or a treated product thereof
10　with N-benzyl-3-pyrrolidinone and a step of harvesting the thus-produced (S)-N-benzyl-3-pyrrolidinol.

In the following, this method is more specifically described. First, the substrate N-benzyl-3-pyrrolidinone, NADPH or a like coenzyme, and a culture of the above
15　transformant and/or a treated product thereof, are added to an appropriate solvent, and the reaction is allowed to proceed under stirring with the pH adjusted. This reaction is carried out at a temperature of 10°C to 70°C, and the pH is maintained at 4 to 10 during the reaction. The reaction
20　can be carried out batchwise or continuously. In the batchwise, the reaction substrate is added to a charge concentration of 0.1% to 70% (w/v). The treated product of the transformant so referred to herein means, for example, a crude extract, cultured cells, lyophilized organism
25　bodies, acetone-dried organism bodies, a disruption product derived therefrom and the like. Further, these can be used in the form of the enzyme itself or cells as such immobilized by known means. This reaction is preferably carried out in the presence of a coenzyme regeneration
30　system. For example, when, in carrying out this reaction, a transformant capable of producing both the polypeptide of the invention and glucose dehydrogenase is used, it is made possible to markedly reduce the consumption of the coenzyme

by further adding glucose to the reaction system.

The (S)-N-benzyl-3-pyrrolidinol produced by the reaction can be harvested by a conventional method. For example, the suspended matter, such as cells, is removed, if necessary, by such treatment as centrifugation or filtration, the reaction solution is made basic by addition of sodium hydroxide or the like and extracted with an organic solvent such as ethyl acetate or toluene, and the organic solvent is then removed under reduced pressure. The product can be purified by further treatment such as distillation or chromatography and so on.

N-Benzyl-3-pyrrolidinone, which is to serve as the substrate in the reaction, can be prepared, for example, by the method described in JP-A-54-16466.

The quantities of N-benzyl-3-pyrrolidinone and (S)-N-benzyl-3-pyrrolidinol can be determined by gas chromatography (column: Uniport B 10% PEG-20M (3.0 mm ID × 1.0 m), column temperature: 200°C, carrier gas: nitrogen, detection: FID). The optical purity of (S)-N-benzyl-3-pyrrolidinol can be measured by high performance liquid chromatography (column: Chiralcel OB (product of Daicel Chemical Industries), eluent: n-hexane/isopropanol/diethylamine = 950/50/1, flow rate: 1 ml/min, detection: 254 nm).

Thus, according to the present invention, it is possible to efficiently produce the polypeptide included in the present invention and, by utilizing the same, an advantageous, production method of (S)-N-benzyl-3-pyrrolidinol is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a chart of the nucleotide sequence of the DNA as determined in Example 3 and the amino acid sequence

deduced therefrom.

Fig. 2 is a chart of the method of constructing the recombinant plasmid pTSBG1 of Example 7 and the structure thereof.

BEST MODES FOR CARRYING OUT THE INVENTION

The following Examples illustrate the present invention in detail. They are, however, by no means limitative of the scope of the present invention.

Detailed procedures and so on concerning the recombinant DNA technology used in the following Examples are described in the following literatures.

Molecular Cloning, 2nd Edition (Cold Spring Harbor Laboratory Press, 1989);

Current Protocols in Molecular Biology (Greene Publishing Associates and Wiley-Interscience).

(Example 1: Purification of enzyme)

An enzyme having the activity in asymmetrically reducing N-benzyl-3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol was uniformly purified from the strain Micrococcus luteus IFO 13867 in the following manner.

(Cultivation of the strain Micrococcus luteus IFO 13867)

A liquid medium (400 mL) having the following composition was prepared in each 2-L Sakaguchi flask and steam-sterilized at 120°C for 20 minutes.

Medium composition:

Trypton	1.6% (w/v)
Yeast extract	1.0% (w/v)
NaCl	0.5% (w/v)
Tap water	
pH 7.0	

This medium was inoculated with 1 ml of a culture fluid of the strain <u>Micrococcus</u> <u>luteus</u> IFO 13867 prepared in advance by preculture in the same medium, and shake culture was carried out at 30°C for 50 hours.

5

(Preparation of cell-free extract)

Cells were collected by centrifugation from the above culture fluid (2 L) and washed with physiological saline. Thus, 42 g of wet cells of the above strain were obtained.

10 These wet cells were suspended in 170 mL of 100 mM phosphate buffer (pH 7.0), then 2-mercaptoethanol and phenylmethylsulfonyl fluoride were added to respective final concentrations of 5 mM and 0.1 mM, and the cells were ultrasonically disrupted using SONIFIRE 250 (product of

15 BRANSON). The cell residues were removed from the disrupted cell disruption by centrifugation, whereby 180 mL of a cell-free extract was obtained.

(Ammonium sulfate fractionation)

20 Ammonium sulfate was added to and dissolved in the cell-free extract obtained in the above manner to attain 40% saturation and the resulting precipitate was removed by centrifugation (during this procedure, the pH of the cell-free extract was maintained at 7.0 using aqueous ammonia).

25 While the pH was maintained at 7.0 in the same manner as in the above procedure, ammonium sulfate was further added to and dissolved in this centrifugation supernatant to attain 65% saturation, and the resulting precipitate was collected by centrifugation. This precipitate was dissolved in 10 mM

30 phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, and the solution was dialyzed overnight using the same buffer.

(Phenyl sepharose column chromatography)

Ammonium sulfate was dissolved in the crude enzyme
solution obtained in the above manner to a final
concentration of 1 M (while the pH of the crude enzyme
solution was maintained at 7.0 using aqueous ammonia), and
the solution was applied to a Phenyl sepharose CL-4B
(product of Pharmacia Biotech) column (130 mL) equilibrated
in advance with 10 mM phosphate buffer (pH 7.0) containing
5 mM 2-mercaptoethanol and 1 M ammonium sulfate so as to
adsorb the enzyme. After washing the column with the same
buffer, the active fraction was eluted with a linear
gradient of ammonium sulfate (from 1 M to 0 M). The active
fraction was collected and dialyzed overnight using 10 mM
phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol.

(DEAE sepharose column chromatography)

The crude enzyme solution obtained in the above
manner was applied to a DEAE sepharose CL-4B (product of
Pharmacia Biotech) column (20 mL) equilibrated in advance
with 10 mM phosphate buffer (pH 7.0) containing 5 mM 2-
mercaptoethanol so as to adsorb the enzyme. After washing
the column with the same buffer, the active fraction was
eluted with a linear gradient of NaCl (from 0 M to 1.0 M).
The active fraction was collected and dialyzed overnight
using 10 mM phosphate buffer (pH 7.0) containing 5 mM 2-
mercaptoethanol.

(Blue Sepharose column chromatography)

The crude enzyme solution obtained in the above
manner was applied to a Blue Sepharose CL-6B (product of
Pharmacia Biotech) column (10 ml) equilibrated in advance
with 20 mM phosphate buffer (pH 6.0) containing 5 mM 2-
mercaptoethanol so as to adsorb the enzyme. After washing

the column with the same buffer, the active fraction was
eluted with a linear gradient of NaCl (from 0 M to 0.5 M).
The active fraction was collected and dialyzed overnight
using 10 mM phosphate buffer (pH 7.0) containing 5 mM 2-
5   mercaptoethanol.

(Gel filtration)
    The crude enzyme solution obtained in the above
manner was applied to a TSK-GEL G3000 SWXL column (product
10 of Tosoh) equilibrated in advance with 100 mM phosphate
buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 100
mM sodium sulfate, and the active fraction was eluted with
the same buffer. The active fraction was collected and
dialyzed overnight using 10 mM phosphate buffer (pH 7.0)
15 containing 5 mM 2-mercaptoethanol to give an
electrophoretically uniform, purified enzyme preparation.
Hereinafter, this enzyme is referred to as BRD.

(Example 2: Measurements of enzyme properties)
20   The enzyme obtained was examined for its
enzymological properties. The enzyme activity was
determined basically by adding the substrate N-benzyl-3-
pyrrolidinone (1 mM), the coenzyme NADPH (0.167 mM) and the
enzyme to 100 mM phosphate buffer (pH 6.5), allowing the
25 reaction to proceed at 30°C for 1 minute and measuring the
decrease in absorbance at the wavelength 340 nm.

(1)   Action:
    It acted on N-benzyl-3-pyrrolidinone with NADPH as
30 the coenzyme and produced (S)-N-benzyl-3-pyrrolidinol with
an optical purity of not less than 99% ee.
(2)   Optimum action pH:
    The enzyme activity was measured by the above method

within the pH range of 4.0 to 7.0 using phosphate buffer and acetate buffer as a buffer. As a result, the optimum pH for the action on N-benzyl-3-pyrrolidinone was found to be 4.5 to 5.5.

5 (3)   Optimum action temperature:

The enzyme activity against the substrate N-benzyl-3-pyrrolidinon exerted for one minute of the reaction was measured within the temperature range of 20°C to 60°C. As a result, the optimum temperature was found to be 40°C to 10   45°C.

(4)   Molecular weight:

The molecular weight of this enzyme was determined by gel filtration using a TSK-GEL G3000 SWXL column (product of Tosoh) and, as the eluent, 100 mM phosphate buffer (pH 15   7.0) containing 5 mM 2-mercaptoethanol and 100 mM sodium sulfate. The molecular weight of the subunit of the enzyme was calculated from the relative mobility relative to reference proteins in SDS-polyacrylamide gel electrophoresis. As a result, the molecular weight of the 20   enzyme was found to be about 29,000 as determined by gel filtration analysis or about 35,000 as determined by SDS-polyacrylamide gel electrophoretic analysis.

(5)   Inhibitors:

The reaction was repeated with the addition of 25   various metal ions and inhibitors shown in Table 1 and, with the activity without addition being taken as 100%, the relative activities upon addition thereof were examined. As shown in Table 1, the enzyme was inhibited by the divalent copper ion.

30

Table 1

Compound	Addition level (mM)	Relative activity (%)
None	–	100
$CoCl_2$	1	99
$CuSO_4$	0.1	6
	1	5
$ZnSO_4$	1	99
$MnCl_2$	1	89
$MgSO_4$	1	99
1,10-Phenanthroline	1	90
5,5-Diphenylhydantoin	0.5	99
EDTA	1	88
PMSF	1	89
PCMB	0.1	78
DTNB	0.01	93
Iodoacetic acid	1	89
NEM	1	94
Quercetin	0.01	94

(Example 3: Cloning of BRD gene)

(Preparation of synthetic oligonucleotide probes)

The purified BRD obtained in Example 1 was digested with bovine pancreas-derived trypsin (product of Wako Pure Chemical Industries), and the amino acid sequences of peptide fragments obtained were determined using ABI 492 model protein sequencer (product of Perkin Elmer). Based on this amino acid sequence, two DNA primers shown under SEQ ID NO:3 and SEQ ID NO:4 in the sequence listing were synthesized in the conventional manner.

(Amplification of BRD gene by PCR)

The chromosomal DNA was extracted from cultured cells of the strain Micrococcus luteus IFO 13867 by the method of Murray et al. (Nucl., Acids Res. 8:4321-4325 (1980)). Then, using the DNA primers prepared as mentioned above, PCR was carried out with the chromosomal DNA obtained as the template, whereupon a DNA fragment (about 250 bp) supposed

to be part of the BRD gene was amplified.

(Construction of chromosomal DNA library)

The chromosomal DNA of the strain <u>Micrococcus</u> <u>luteus</u>
IFO 13867 was completely digested with the restriction
enzyme BamHI, followed by separation by agarose gel
electrophoresis. Then, using the DNA fragment obtained in
the above manner (about 250 bp) as the probe, the digest of
the chromosomal DNA was analyzed by the Southern method (J.
Mol. Biol., 98, 503 (1975)) (the labeling of the DNA probe
and detection thereof being carried out using the Gene
Images labeling/detection system (product of Amersham)).
As a result, a DNA fragment of about 4.5 kb was found to
hybridize with the above DNA probe.

Therefore, the above digest was subjected to
separation by agarose gel electrophoresis, and 4.3 kb to
6.2 kb DNA fragments were recovered. These DNA fragments
were inserted into the vector plasmid pUC19 (product of
Takara Shuzo) at the BamHI site thereof, followed by
introduction into the strain <u>Escherichia</u> <u>coli</u> JM109
(product of Takara Shuzo). A chromosomal DNA library of
this strain was thus constructed.

(Screening of the chromosomal DNA library)

Using the DNA fragment obtained in the above manner
as the probe, the chromosomal DNA library constructed in
the above manner was subjected to screening by the colony
hybridization method (the labeling of the DNA probe and
detection thereof being carried out using the Gene Images
labeling/detection system (product of Amersham) and the
experimental procedure being performed according to the
manual attached to the system). As a result, one positive
colony was obtained. Therefore, a recombinant plasmid pUC-

BB, produced by insertion of the DNA (about 4.5 kb) obtained from this positive colony was selected as a BRD gene-containing chromosomal DNA clone.

5 (Determination of nucleotide sequence)

The recombinant plasmid pUC-BB obtained in the above manner was treated with various restriction enzymes and the resulting digestion fragments were analyzed, and a restriction enzyme cleavage map thereof was prepared. Then,
10 recombinant plasmids were constructed by inserting various DNA fragments obtained on the occasion of the above analysis into pUC19 at the multicloning site thereof. Using these recombinant plasmids, the nucleotide sequences of each inserted fragment was analyzed using an ABI PRISM
15 Dye Terminator Cycle Sequencing Ready Reaction Kit (product of Perkin Elmer) and ABI 373A DNA Sequencer (product of Perkin Elmer), and the nucleotide sequence of a DNA fragment (about 1.4 kb) supposed to contain the target gene was determined. That nucleotide sequence is shown in Fig.
20 1. As for the structural gene portion in this nucleotide sequence, the amino acid sequence deduced from that nucleotide sequence is shown below the nucleotide sequence in Fig. 1. As a result of comparison of this amino acid sequence with the partial amino acid sequence of digested
25 fragments of purified BRD by trypsin, the whole partial amino acid sequence of purified BRD was found to exist in the amino acid sequence deduced from the nucleotide sequence and to be quite identical in that portion (the underlined amino acid sequence in Fig. 1). Thus, that gene
30 was judged to be the BRD gene.

(Example 4: Construction of BRD gene-containing recombinant plasmid)

A double-stranded DNA resulting from addition of an
NdeI site to the initiation codon portion of the structural
gene of BRD and further addition, just behind the 3'
terminus thereof, of a termination codon (TAA) and an EcoRI
5   cleavage point was obtained in the following manner. Based
on the nucleotide sequence determined in Example 3, an N-
terminal DNA primer with an NdeI site added to the
initiation codon part of the BRD gene and a C-terminal DNA
primer with a termination codon (TAA) and an EcoRI site
10   added just behind the 3' terminus of the same gene were
synthesized. The nucleotide sequences of these two primers
are shown under SEQ ID NO:5 and SEQ ID NO:6 in the sequence
listing. Using these two synthetic DNA primers, together
with the plasmid pUC-BB obtained in Example 3 as the
15   template, a double-stranded DNA was amplified by PCR. The
DNA fragment obtained was digested with NdeI and EcoRI, and
the resulting fragment was inserted into the plasmid pUCNT
(WO 94/03613) at the NdeI-EcoRI site downstream of the lac
promoter to give a recombinant plasmid pNTBR.
20

(Example 5: Addition of Shine-Dalgarno sequence to a site
upstream of the BRD gene)
     For attaining high level expression of the BRD gene
in Escherichia coli, a plasmid was obtained from the
25   plasmid pNTBR prepared in Example 4 by newly adding the
E.coli-derived Shine-Dalgarno sequence (9 bases) to a site
upstream of the initiation codon of the same gene, as
follows. First, a plasmid pUCT was constructed by
converting G in the NdeI site of the E.coli expression
30   vector pUCNT used in Example 4 to T by the PCR method.
Then, an N-terminal DNA primer resulting from addition of
the E.coli -derived Shine-Dalgarno sequence (9 bases) at 5
bases upstream of the initiation codon of the BRD gene

shown under SEQ ID NO:2 in the sequence listing and further addition of an EcoRI site at just before the Shine-Dalgarno sequence and a C-terminal DNA primer resulting from addition of a SacI site just behind the 3' terminus of the

5 same gene were synthesized in the conventional manner. The nucleotide sequences of these two primers are shown under SEQ ID NO:7 and SEQ ID NO:8 in the sequence listing. Using these two DNA primers, with the plasmid pNTBR constructed in Example 4 as the template, a double-stranded DNA was

10 synthesized by PCR. The DNA fragment obtained was digested with EcoRI and SacI, and the resulting fragment was inserted into the plasmid pUCT at the EcoRI-SacI site (downstream of the lac promoter) to give a recombinant plasmid pTBH.

15

(Example 6: Reduction in GC ratio in BRD gene)
    For further attaining high level expression of the BRD gene in E.coli, a plasmid pTSBH was constructed by substituting a DNA lower in GC ratio for the segment from

20 the 1st to 118th base of the same gene in the plasmid pTBH constructed in Example 5, without altering the amino acid sequence coded thereby, as follows.
    A double-stranded DNA having the sequence shown under SEQ ID NO:9 in the sequence listing was prepared by the

25 conventional method. This was digested with EcoRI and XhoI, and a plasmid pTSBH substituted for the DNA fragment detached from pTBH by digestion with the same restriction enzymes and containing a 5' terminal portion of the BRD gene was obtained.

30

(Example 7: Construction of recombinant plasmid containing both BRD gene and glucose dehydrogenase gene)
    A double-stranded DNA resulting from addition of the

E.coli -derived Shine-Dalgarno sequence (9 bases) at 5
bases upstream of the initiation codon of the strain
Bacillus megaterium IAM 1030-derived glucose dehydrogenase
(hereinafter referred to as GDH) gene, of a SacI cleavage

5   point just before the above sequence and of a BamHI
cleavage point just behind the termination codon was
prepared in the following manner. Based on the nucleotide
sequence information about the GDH gene, an N-terminal DNA
primer resulting from addition of the E.coli -derived

10   Shine-Dalgarno sequence (9 bases) at 5 bases upstream of
the initiation codon of the structural gene of GDH and
further addition of a SacI cleavage point just before the
above sequence, and a C-terminal DNA primer resulting from
addition of a BamHI site just behind the termination codon

15   of the structural gene of GDH were synthesized by the
conventional method. The nucleotide sequences of these two
primers are shown under SEQ ID NO:10 and SEQ ID NO:11,
respectively, in the sequence listing. Using these two DNA
primers, together with the plasmid pGDK1 (Eur. J. Biochem.

20   186, 389 (1989)) as the template, a double-stranded DNA was
synthesized by PCR. The DNA fragment obtained was digested
with SacI and BamHI and inserted into the SacI-BamHI site
(occurring downstream of the BRD gene) of the pTSBH
constructed in Example 5 to give a recombinant plasmid

25   pTSBG1. The method of constructing pTSBG1 and the
structure thereof are shown in Fig. 2.

(Example 8: Production of recombinant E.coli)
        E.coli HB101 (product of Takara Shuzo) was

30   transformed using the recombinant plasmids pTBH, pSTBH and
pTSBG1 obtained in Examples 5, 6 and 7, to give recombinant
E.coli HB101 (pTBH), HB101 (pSTBH) and HB101 (pTSBG1),
respectively. Among the thus-obtained transformants,

E.coli HB101 (pTSBH) and HB101 (pTSBG1) have been deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary (Address: Central 6, 1-1 Higashi 1-chome, Tsukuba City, Ibaraki Prefecture, Japan) under the accession number FERM BP-7118 (deposition date: April 11, 2000) and the accession number FERM BP-7119 (deposition date: April 11, 2000), respectively.

Further, the plasmid pGDA2 (J. Biol. Chem., (1989), 264, 6381) was double-digested with EcoRI and PstI and the thus-obtained DNA fragment (about 0.9 kb) containing the Bacillus megaterium IWG3-derived GDH gene was inserted into the plasmid pSTV28 (product of Takara Shuzo) at the EcoRI-PstI site thereof to construct a recombinant plasmid pSTVG. E.coli HB101 (pTSBH) made competent in advance by the calcium chloride method was transformed with this plasmid pSTVG at a high rate of introduction. Thus, E.coli HB101 (pTSBH, pSTVG) was obtained with ease.

(Example 9: BRD expression in recombinant E.coli)

The recombinants E.coli HB101 (pTBH) and HB101 (pTSBH) obtained in Example 8 were each shake-cultured on 2 X YT medium containing 200 µg/ml of ampicillin at 28°C for 15 hours. A 1-ml portion of this preculture fluid was inoculated into 100 ml of a medium sterilized by autoclaving in a 500-ml Sakaguchi flaks and comprising 1.5% (w/v) glycerol, 1.5% (w/v) Bacto tryptone, 0.4% (w/v) Bacto yeast extract, 0.2% (w/v) sodium chloride, 0.8% (w/v) potassium dihydrogen phosphate, 0.05% (w/v) magnesium sulfate heptahydrate, and 0.033% (w/v) Adekanol LG109 (product of Asahi Denka Kogyo), as adjusted to pH 6.0, and shake culture was carried out at 30°C for 60 hours. Cells were harvested from such culture fluids by using a

centrifuge, then suspended in 100 mM phosphate buffer (pH
6.5) and ultrasonically disrupted to give a cell-free
extract.

5 The BRD activity of this cell-free extract was
determined in the following manner. Thus, the BRD activity
was determined by adding the substrate N-benzyl-3-
pyrrolidinone (1 mM), the coenzyme NADPH (0.167 mM) and the
enzyme to 100 mM phosphate buffer (pH 6.5) and measuring
the decrease in absorbance at the wavelength 340 nm at 30°C.
10 The enzyme activity capable of oxidizing 1 µmol of NADPH to
NADP in 1 minute under these reaction conditions was
defined as 1 unit. Thus-determined BRD activities of the
cell-free extracts were expressed in terms of specific
activity and compared with that of the transformant holding
15 the vector plasmid pUCNT. Comparison was also made with
the BRD activity of a cell-free extract derived from the
strain <u>Micrococcus luteus</u> IFO 13867 as prepared in the same
manner as in Example 1. The results thus obtained are
shown in Table 2.

20

Table 2

Name of strain	Specific BRD activity (U/mg)
E.coli HB101(pUCNT)	<0.01
E.coli HB101(pTBH)	0.06
E.coli HB101(pTSBH)	0.61
Micrococcus luteus IFO 13867	0.06

As for <u>E.coli</u> HB101 (pTSBH), a distinct increase in
BRD activity was observed as compared with <u>E.coli</u> HB101
25 (pUCNT) which is transformed with the vector plasmid alone
and, when compared with the strain <u>Micrococcus luteus</u> IFO
13867, the activity was about 10-fold higher.

(Example 10: Simultaneous expression of BRD and GDH in recombinant E.coli)

The recombinant E.coli HB101 (pTSBG1) and HB101 (pTSBH, pSTVG) obtained in Example 8 were cultured and treated in the same manner as in Example 9 to give the respective cell-free extracts, which were assayed for GDH activity in the following manner. The GDH activity was determined by adding the substrate glucose (0.1 M), the coenzyme NADP (2 mM) and the enzyme to 1 M Tris hydrochloride buffer (pH 8.0) and measuring the increase in absorbance at the wavelength 340 nm at 25°C. The enzyme activity capable of reducing 1 µmol of NADP to NADPH in 1 minute under these reaction conditions was defined as 1 unit. The BRD activity was also determined in the same manner as in Example 9. Thus-determined BRD and GDH activities of the cell-free extracts were each expressed in terms of specific activity and compared with those of E.coli HB101 (pTSBH) and HB101 (pUCNT) which is transformed with the vector alone. The results are shown in Table 3.

Table 3

Name of strain	Specific BRD activity (U/mg)	Specific GDH activity (U/mg)
E.coli HB101(pUCNT)	<0.01	<0.01
E.coli HB101(pTSBH)	0.61	<0.01
E.coli HB101(pTSBG1)	0.52	89
E.coli HB101(pTSBH,pSTVG)	0.69	3.2

As for E.coli HB101 (pTSBG1) and HB101 (pTSBH, pSTVG), distinct increases in BRD activity and GDH activity were observed as compared with E.coli HB101 (pUCNT) which is transformed with the vector plasmid alone.

(Example 11: Synthesis of (S)-N-benzyl-3-pyrrolidinol from N-benzyl-3-pyrrolidinone using recombinant E.coli produced by introduction of BRD gene)

5   The culture fluid of the recombinant E.coli HB101 (pTSBH) obtained in Example 9 was ultrasonically disrupted using SONIFIRE 250 (product of BRANSON). To 25 ml of this cell disruption fluid were added 1,350 U of glucose dehydrogenase (product of Amano Pharmaceutical), 3.0 g of

10   glucose, 3.0 mg of NADP and 0.25 g of N-benzyl-3-pyrrolidinone. While this reaction mixture was stirred at 30°C with pH adjusted to 6.5 using 5 M hydrochloric acid or sodium hydroxide, N-benzyl-3-pyrrolidinone was added thereto at an interval of 0.25g/hour. After addition of a

15   total of 2.0 g of N-benzyl-3-pyrrolidinone, stirring was further continued for 20 hours. After completion of the reaction, 2.5 ml of a 5 M aqueous solution of sodium hydroxide was added, the mixture was extracted with toluene, and the solvent was removed. Analysis of the resulting

20   extract revealed that N-benzyl-3-pyrrolidinol was obtained in 74% yield. The N-benzyl-3-pyrrolidinol produced on that occasion was the S form with an optical purity of not less than 99% ee.

       The quantity of N-benzyl-3-pyrrolidinone and N-

25   benzyl-3-pyrrolidinol was determined by gas chromatography (column: Uniport B 10% PEG-20 M (3.0 mm ID x 1.0 m), column temperature: 200°C, carrier gas: nitrogen, detection: FID). The optical purity of (S)-N-benzyl-3-pyrrolidinol was determined by high performance liquid chromatography

30   (column: Chiralcel OB (product of Daicel Chemical Industries), eluent: n-hexane/isopropanol/diethylamine = 950/50/1, flow rate: 1 ml/min, detection: 254 nm).

(Example 12: Synthesis of (S)-N-benzyl-3-pyrrolidinol from
N-benzyl-3-pyrrolidinone using recombinant E.coli capable
of simultaneous expression of BRD and glucose
dehydrogenase)

5   To 25 ml of the culture fluid of the recombinant
E.coli HB101 (pTSBG1) obtained in Example 9 were added 2.5
g of glucose, 3.0 mg of NADP and 0.25 g of N-benzyl-3-
pyrrolidinone. While this reaction mixture was stirred at
30°C with pH adjusted to 6.5 using 5 M hydrochloric acid or
10   sodium hydroxide, N-benzyl-3-pyrrolidinone was added
thereto at an interval of 0.25g/2 hours.   After addition
of a total of 1.0 g of N-benzyl-3-pyrrolidinone, stirring
was further continued for 17 hours. After completion of
the reaction, 1.2 ml of a 5 M aqueous solution of sodium
15   hydroxide was added, the mixture was extracted with toluene,
and the solvent was removed. Analysis of the resulting
extract revealed that N-benzyl-3-pyrrolidinol was obtained
in 92% yield. The N-benzyl-3-pyrrolidinol produced on that
occasion was the S form with an optical purity of not less
20   than 99% ee.

(Example 13: Synthesis of (S)-N-benzyl-3-pyrrolidinol from
N-benzyl-3-pyrrolidinone using recombinant E.coli capable
of simultaneous expression of BRD and glucose
25   dehydrogenase)

          To 25 ml of the culture fluid of the recombinant
E.coli HB101 (pTSBH, pSTVG) obtained in Example 9 were
added 2.5 g of glucose, 3.0 mg of NADP and 0.25 g of N-
benzyl-3-pyrrolidinone. While this reaction mixture was
30   stirred at 30°C with pH adjusted to 6.5 using 5 M
hydrochloric acid or sodium hydroxide, N-benzyl-3-
pyrrolidinone was added thereto at an interval of
0.25g/hour. After addition of a total of 2.0 g of N-

benzyl-3-pyrrolidinone, stirring was further continued for
16 hours. After completion of the reaction, 2.5 ml of a 5
M aqueous solution of sodium hydroxide was added, the
mixture was extracted with toluene, and the solvent was
5   removed. Analysis of the resulting extract revealed that
N-benzyl-3-pyrrolidinol was obtained in 93% yield. The N-
benzyl-3-pyrrolidinol produced on that occasion was the S
form with an optical purity of not less than 99% ee.

10   INDUSTRIAL APPLICABILITY
       As a result of cloning of a gene of a polypeptide
having enzyme activity in asymmetrically reducing N-benzyl-
3-pyrrolidinone to produce (S)-N-benzyl-3-pyrrolidinol and
analysis of the nucleotide sequence thereof, it has become
15   possible to obtain a transformant capable of producing, at
high levels, the above polypeptide. It has also become
possible to obtain a transformant capable of producing, at
high levels, the polypeptide and glucose dehydrogenase
simultaneously. Furthermore, it has become possible to
20   efficiently synthesize (S)-N-benzyl-3-pyrrolidinol from N-
benzyl-3-pyrrolidinone using such transformants.